Paleoproterozic Icehouses and the Evolution of Oxygen Mediating Enzymes: The Case for a Late Origin of Photosystem-II

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Two major geological problems regarding the origin of oxygenic photosynthesis are: (1) identifying a source of oxygen predating biological oxygen production and capable of driving the evolution of oxygen tolerance, and (2) determining when oxygenic photosynthesis evolved. One solution to the first problem is the accumulation of photochemically-produced H2O2 at the surface of glaciers and its subsequent incorporation into ice. Melting at the glacier base would release H2O2, which interacts with seawater to produce O2 in an environment shielded from the lethal levels of ultraviolet radiation needed to produce H2O2. Answers to the second problem are controversial and range from 3.8 to 2.2 Ga. A skeptical view, based on metals that have redox potentials close to oxygen, argues for the late end of the range. The preponderance of geological evidence suggests little or no oxygen in the late Archaean atmosphere (< 1 ppm). The main piece of evidence for an earlier evolution of oxygenic photosynthesis comes from lipid biomarkers. Recent work, however, has shown that 2-methylhopanes, once thought to be unique biomarkers for cyanobacteria, are also produced anaerobically in significant quantities by at least two strains of anoxygenic phototrophs. Sterane biomarkers provide the strongest evidence for a date ≥2.7 Ga but could also be explained by the common evolutionary pattern of replacing anaerobic enzymes with oxygen-dependent ones. Although no anaerobic sterol synthesis pathway has been identified in the modern biosphere, enzymes that perform the necessary chemistry do exist. This analysis suggests that oxygenic photosynthesis could have evolved close in geological time to the Makganyene Snowball Earth Event and argues for a causal link between the two.

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1. INTRODUCTION

The debate about the history of atmospheric oxygen is most likely the longest-running, still unresolved controversy in the history of modern science. It began in the mid-nineteenth century with some of the earliest publications in biogeochemistry, when Jacques Joseph Ebelman (1845) and Karl Gustav Bischof (1854), considering the balance of oxygen release by organic carbon and pyrite burial and oxygen consumption by iron and manganese oxidation, speculated that atmospheric oxygen levels may have changed over time with changes in biota (Berner & Maasch 1996). In 1856, C. J. Koene (1856/2004) argued, based on the presence of reduced matter in early rocks, that the Earth's initial atmosphere was high in carbon dioxide and free of oxygen, and that over geological time the action of photosynthetic plants had resulted in a decline in carbon dioxide and a rise of oxygen. These early works apparently fell upon deaf ears, as few successor publications appeared until late in the nineteenth century (see Stevenson (1900), van Hise (1904), and Clarke (1924) for reviews). Koene's work had by this time vanished into obscurity; Stevenson (1900) knew of it only through a synopsis published in 1893-1894 by T. L. Phipson, one of Koene's former students.

Later, the Rhodesian geologist A. M. MacGregor (1927) complemented the chemical models with observations, both in the field and in the laboratory, of the Precambrian of southern Africa. He argued that the quantity of carbon buried in shales was sufficient to account for the quantity of oxygen in the atmosphere and therefore suggested that oxygen had accumulated over the course of Earth's history. Consequently, he wrote, "the assumption [that the most ancient rocks were themselves formed under an atmosphere of oxygen] can only be justified by the clearest field evidence of contemporary oxidation in the most ancient rocks themselves" (p. 158).

MacGregor turned to the rocks of Rhodesia and found evidence that the pre-Lomagundi metasediments of the Archaean Basement Schists, as he called them, were deposited under an oxygen-free atmosphere. In particular, he noted that: (1) chemical analyses showed high ferrous/ ferric ratios in Archaean sediments; (2) the deposition of banded ironstones and their confinement to the Precambrian could be explained if they were formed by the action of either iron-oxidizing bacteria or oxygen-producing organisms in an anaerobic ocean; (3) rounded pyrite-bearing clasts in Archaean conglomerates suggested fluvial transport without exposure to oxidizing conditions.

The post-World War II era saw the addition of more sophisticated chemical theory and measurements to the discussion. Urey (1952) introduced considerations of equilibrium thermodynamics and discussed some of the earliest sulfur isotope evidence. Holland (1962) expanded Urey's calculations and considered, among other lines of evidence, the conditions necessary to oxidize uranium and prevent the deposition of detrital uraninite. By the mid-1960s, many of the arguments raised in modern discussions of the timing of the rise of oxygen were in place. In 1965, the National Academies of Science hosted one of the first symposia devoted to the issue (Cloud et al. 1965).

The past 42 years have seen numerous revolutions in geology and biology – from the molecular revolution in microbiology, to the development of theories of plate tectonics and abrupt climate change, to the recognition of the role of non-uniformitarian events in Earth history – as well as the development of at least one new technique, the study of mass-independent fractionation (MIF) of sulfur isotopes, that may directly reveal anoxia in the ancient atmosphere (Farquhar et al. 2000). Combined with high-resolution data of critical intervals in Earth history from ongoing continental drilling projects, these advances hold out the promise of a fourth stage in our understanding of the evolution of oxygenic photosynthesis and the rise of oxygen – one that can build a strong connection between the predictions of theory and specific geological observations.

In this paper, we first review a recent development that provides a solution to a wlong-standing "chicken-and-the-egg" problem concerning the evolution of PS II: that O_2-evolving processes require O_2-mediating enzymes to limit toxicity, while O_2-mediating enzymes are unlikely to evolve without a source of O_2. A similar problem arises from the fact that cholrophyll synthesis in green plants has 3 steps that require O_2, which is produced by oxygenic photosynthesis. Next, we review the biomarker controversy, which arises from the apparent conflict between pieces of geological evidence for planetary anoxia contemporary with lipid biomarkers interpreted as suggesting the existence of oxygenic photosynthesis. Finally, we will

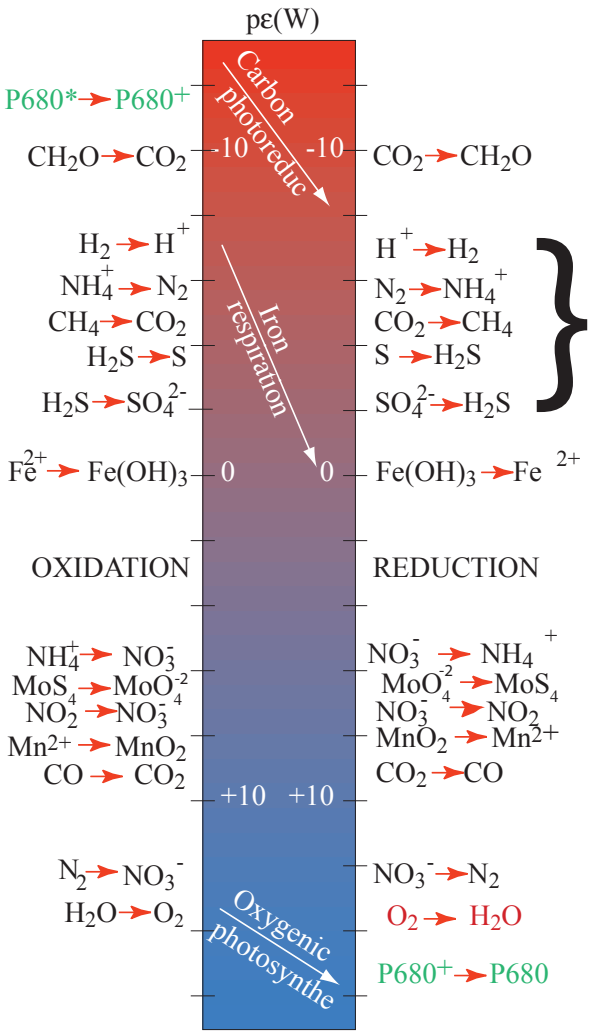

Figure 1. Redox couples in water at pH 7 and 25° C (adapted from Gaidos et al. 1999). Half-reactions on the left couple spontaneously with those below them on the right, and most pairs are suitable for driving biological metabolism. The primary donor of Photosystem II (P680) has the largest redox potential change known for any organic molecule, and in its oxidative (rest) state, it is capable of oxidizing water, producing oxygen. The heavy brackets signify the range of gases emitted from volcanic eruptions. Subaerial eruptions tend to be more oxidizing than subaqueous eruptions, so the shift in eruptive style towards subaerial vulcanism noted by Kump & Barley (2007) would have helped the eventual oxidation of the atmosphere, even though all volcanic gases are on the reducing side of the ferrous/ferric couple. Note that the term 'oxidizing' appears to have quite separate meanings in the geological and biological sciences. Redox couples near the Quartz-Fayalite-Magnetite (QFM) buffer in rocks are considered 'oxidizing' by Earth scientists, but are actually on the reducing side of the diagram and clearly far away from the NO_3^- and O_2 couples considered oxidizing by biologists.

summarize a simple and plausible sequence of events that, in our view, accounts for all of the biological and geological constraints on the geological history of oxygenic photosynthesis.

2. GLACIAL PEROXIDES AND THE EVOLUTION OF OXYGEN MEDIATING ENZYMES.

The ability of dissolved O_2 to accept an electron from a suitable donor (e.g., Fe^{+2}) and form the superoxide radical, O_2^-, is a severe threat to organisms living in an aerobic environment. The superoxide radical reacts spontaneously with water to form the hydroxyl radical (•OH), which attacks the sugar/phosphate backbone of DNA. The need to control this toxic process fostered the evolution of O_2-mediating enzymes, such as superoxide reductase (Jenney et al. 1999) and superoxide dismutase (which convert O_2^- to H_2O_2) (Wolfe-Simon 2005), catalase (which converts H_2O_2 to water), and various oxygen binding domains like heme that help prevent superoxide formation (Niyogi 1999). O_2-mediating pathways had to evolve prior to metabolic utilization of O_2, including the production of oxygen by the O_2-evolving complex of Photosystem II (PS-II). Moreover, the O_2-evolving complex appears to derive from the Mn cluster of catalase, also implying that the O_2-mediating enzymes came first (Blankenship & Hartman 1998; McKay & Hartman 1991).

But while there must be a source of superoxide radicals to act as an adaptive pressure for the evolution of oxygen-mediating pathways, most geological and geochemical processes in Earth's atmosphere and hydrosphere are relatively reducing (Figure 1). Volcanic gases are buffered by redox reactions on the reducing end of this spectrum, more reducing than the ferrous-ferric redox couplet and far from the highly oxidized water/oxygen couplet. In fact, photolytic reactions involving ultraviolet (UV) radiation and water vapour provide the only known abiotic pathway for producing biologically significant concentrations of molecular O_2. However, the environments under which this processes can happen are lethal to all living organisms, as the same UV radiation is destructive to complex organic molecules, including DNA.

One solution to this puzzle is provided by Liang et al., (2006), who note that Antarctic ice cores preserve interannual variations in H_2O_2 concentration that reflect the history of the

Antarctic ozone hole (Frey et al. 2006; Frey et al. 2005). As ozone is the major filter for short-wavelength UV, reductions in stratospheric ozone facilitate photochemical reactions involving H_2O that generate H_2O_2 and H_2 gas. H_2 diffuses away and is lost, whereas H_2O_2, with a freezing point near that of water, condenses out and accumulates in the ice.

Liang et al. (2006) noted that the late Archaean Pongola glaciation and early Paleoproterozoic glaciations occurred in atmospheres with little oxygen, lacking an ozone screen and bathed in UV radiation strong enough to produce the MIF fractionation observed in sulphur compounds (Figure 2). During these glaciations, the same photochemical processes acting today in Antarctica would have acted over entire ice sheets, building up H_2O_2 concentrations. While similar photochemistry would occur in the liquid ocean, H_2O_2 produced there would diffuse away rather than accumulating. During a normal (non-Snowball) glaciation, peroxide-laced snow would follow normal glacial dynamics, being compressed into glacial ice, flowing in glaciers to the ocean, and melting either there or along the wet-base portions. Upon melting, H_2O_2 would have disproportionated into O_2 and water ($2\ H_2O_2 \rightarrow 2H_2O + O_2$), thereby producing an environment protected from lethal UV radiation but 'poisoned' with trace amounts of oxygen, in which oxygen-mediating enzymes might evolve. The small 'whiffs of oxygen' reported recently by Anbar et al.(2007) and Kaufmann et al. (2007) at two nearby localities on the paleocontinent of Vaalbarra at around 2.50 Ga could be the geochemical fingerprint of oxic meltwaters mixing with glacial flour (powdered rock) at the base of a polar ice field. Although the oldest confirmed glacial unit in the Paleoproterozoic occurs sometime after 2.45 Ga (Evans 2000; Young et al. 2001), we do not know the full duration of the late Archaean and Paleoproterozoic glacial epochs, as the preservation of glacial deposits depends upon having continents in the correct position, having sufficient accomodation space, and the fate of deposits over geological time. Earth may well have experienced glacial Icehouse conditions, akin to those of the late Paleozoic and Cenozoic, during the time gap between the preserved Pongola and Huronian glacial deposits.

3. ARE ARCHAEAN LIPID BIOMARKERS CONTAMINANTS?

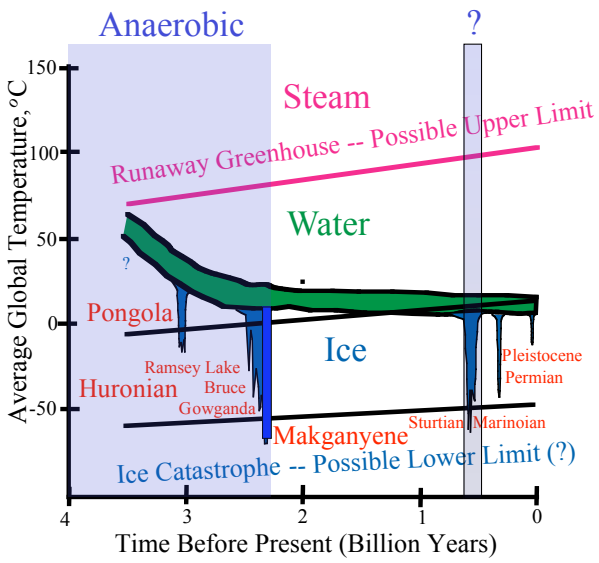

Figure 2. Earth's glacial history, with intervals of atmospheric anoxia indicated by the light shading (adapted from a cartoon of Lovelock, 1979). Major Precambrian glaciations are indicated by 'icicles' dangling from a temperature vs. time curve through Earth history, and include the Pongola of southern Africa at ~ 2.9 Ga, the Paleoproterozoic glaciations between ~2.5 – 2.22 (the three major units of the Huronian series in Laurentia and the Makganyene of southern Africa), the Cryogenian glaciations in the Neoproterozoic (ca. 800 – 600 Ma), and the Permian and Neogene glaciations in the Phanerozoic. The UV-peroxide generating mechanism of Liang et al. (2006) is expected to operate on any ice sheet formed in an atmosphere without an ozone or other UV screen. The '?' signifies the possibility of earlier, not-yet-recognized glacial intervals.

As of 2004, at least 473 biochemical reactions were known in which molecular O_2 was a substrate (Raymond & Blankenship 2004; Raymond & Segre 2006). Hence, one way to constrain the history of oxygen is to look in sediments for biomarkers that are the products of oxygen-dependent reactions. As with any geobiological tracer, one must verify that the materials being studied are the same age as the rock; and as with many promising new techniques, the application of organic geochemistry to the Precambrian had a rocky, controversial start. During the 1960s and early 1970s, a flurry of studies reported trace levels of Precambrian porphyrins, fatty acids, alkanes, acyclic isoprenoids, and even amino acids (Brocks et al. 2003a; Hayes et al. 1983). Follow-up studies revealed that virtually all the organic compounds were contaminants, usually petroleum-derived hydrocarbons from the laboratory and field environments. In response, and to their great

credit, workers in the field evolved into their own most severe critics.

Despite these discouraging initial problems, great progress has been made in identifying the proper target molecules to study and in elucidating the diagenetic processes operating on them (Knoll et al. 2007). The lipid fraction from biological membranes is by far the best, as lipids can preserve the topology of their skeletal backbones despite various changes during diagenesis (like the loss and replacement of hydroxyl groups, linear side chain degradation, and saturation of double bonds). Of particular importance as potential constraints on the history of oxygen are the degradation products of polycyclic isoprenoids, especially hopanols and sterols, which preserve enough of their structural information to identify the biochemical pathways through which they formed. Although not unknown, production of the same isoprenoid compound through different pathways is rare. The Precambrian fossil record of hopane and sterols have been the primary focus of a series of papers (Brocks et al. 2003a; Brocks et al. 2003b; Brocks et al. 1999; Summons et al. 2006; Summons et al. 1999). For background, we recommend two excellent reviews on sterol biosynthesis and the structure and function of the family of triterpene cyclase enzymes, Lesburg et al. (1998) and Wendt et al. (2000), and the oxygen dependence for sterol synthesis in yeast by Rosenfeld & Beauvoit, (2003).

This body of work employs vastly superior techniques and internal controls for reproducibility than were used in the discredited studies of the 1960s. However, concerns about contamination have not evaporated, as Brocks (2003b, p. 4322) noted: *"The age of the molecules that contain the biologic information is not fully resolved. Hence, paleobiological interpretation ... should be cited cautiously and with reference to the remaining uncertainty of syngeneity."* Possible 'red flags' include the presence of characteristically Phanerozoic biomarkers (such as dinosteranes, generally associated with dinoflagellates, a Mesozoic-Cenozoic taxon), as well as the puzzling lack of any geological trace of intermediate evolutionary stages in the long sterol biosynthetic pathway. Pearson et al. (2003) identified one extant Planctomycete, *Gemmata obscuriglobus*, which has just such an intermediate sterol synthesis pathway and is possibly on the ancestral eukaryote lineage (Fuerst 2005), but it is unknown whether the pathway is an immature relict or has been truncated. Regardless, as Brocks (2005) notes, the incremental pattern of eukaryotic radiation observed in the Proterozoic fossil record ought to be matched by similar changes in lipid biomarkers, but instead Archaean sterols with a fully modern fingerprint materialize nearly a billion years before the first fossils with possible eukaryotic affinity. New analytical methods, such as the examination of hydrocarbons in fluid inclusions (Dutkiewicz et al. 2006; Ueno et al. 2006), may soon resolve this contamination controversy.

4. IS O_2 A FUNDAMENTAL REQUIREMENT FOR HOPANE & STEROL FORMATION?

For organic biomarkers to provide strong constraints on paleo-oxygen levels, it is important to verify that the fundamental chemistry involved requires oxygen, precluding any possibility of anaerobic mechanisms, and that the basic processes on which the inferences are based have not changed over geological time. Even if biosynthetic pathways leading to hopanol and sterol synthesis evolved during Hadean or Archaean time, we argue that recent work has weakened the case considerably linking them to the presence of oxygenic photosynthesis. Brocks et al. (2003b, p. 4331) summarize their four lines of molecular evidence for free O_2 in the surface waters as follows:

"1. The bitumens contain molecular fossils of bacteriohopanoids. Although hopanoid biosynthesis does not require oxygen, these lipids have never been isolated from strict anaerobes (Ourisson et al., 1987).

"2. The Archaean shales also contain high relative concentrations of cyanobacterial 2 - methylhopanes. These biomarkers are indirect evidence for oxygen release within the photic zone."

"3. The side-chain degradation pattern of the 2–methylhopane series (Fig. 5) indicates oxic conditions during earliest diagenesis of cyanobacterial organic matter."

"4. The bitumens contain molecular fossils of sterols. Sterol biosynthesis in extant eukaryotes requires dissolved oxygen in concentrations

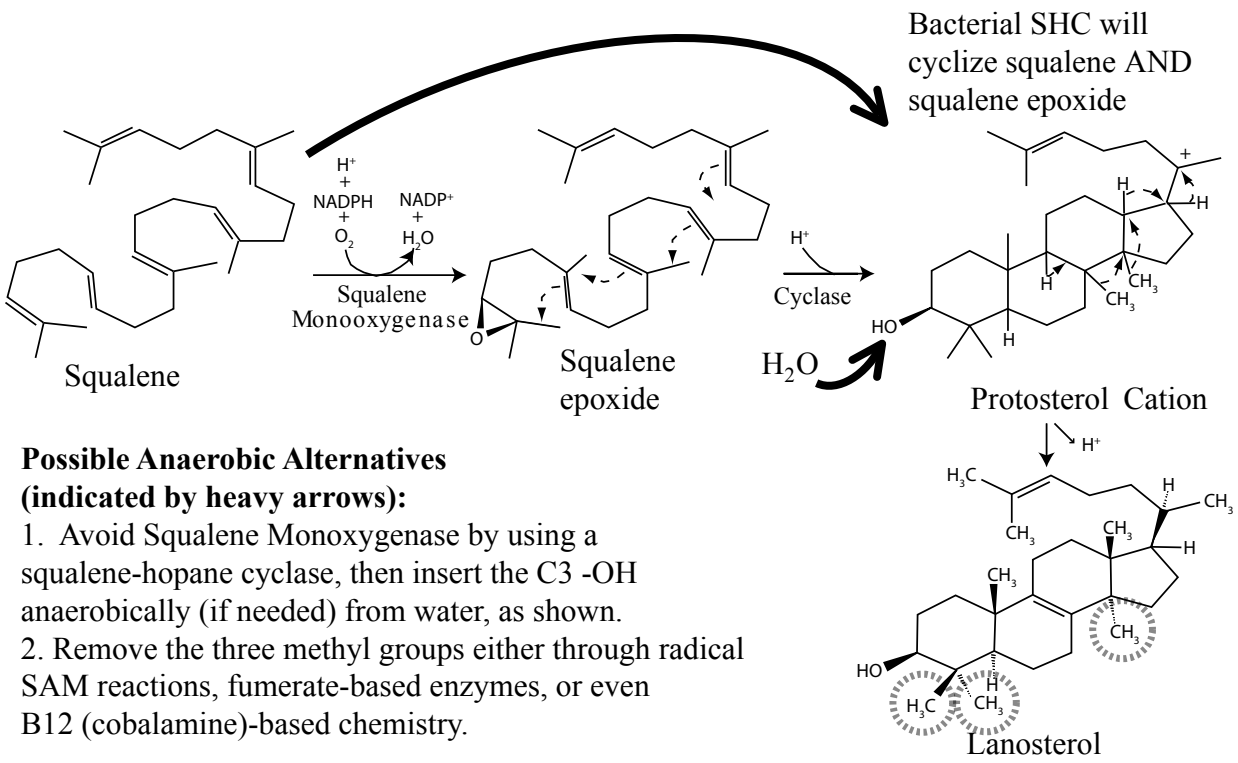

Fig. 3. Sterol biosynthetic pathway, with proposed modifications for anoxic operation. In eukaryotes, the oxygen dependent steps include the conversion of squalene to squalene epoxide, and the removal of two methyl groups on the C4 carbon and one on the C14 carbon (indicated by faint red circles). As indicated by the long, heavy arrow, we suggest that an ancestral anaerobic eukaryote may have directly done the initial cyclization reaction on squalene rather than its epoxide, in the fashion of many bacterial squalene-hopane cyclase (SHC) enzymes that will cyclize either form. The C3 hydroxyl (if it was really there in Archaean time) could be added after the cyclization reaction as described in the text. Similarly, a variety of demethylation reactions are known from sulphate reducing organisms that could, in principle, remove the three methyl groups from lanosterol to form ergesterol (not shown), which is the minimal sterol from which eukaryotes can grow anaerobically.

equivalent to 1% PAL (Jahnke and Klein, 1983)."

Recent work suggests that all of these can be explained by mechanisms that do not require free oxygen in the environment; each will be considered in turn:

Bacteriohopanoids and 2-methylhopanes. As Brocks et al. (2003b) note, production of hopanoids does not involve O_2, and subsequent work has identified multiple anaerobic bacteria that produce hopanoids. Fischer et al. (2005) found that at least one bacterium, *Geobacter sulphurreducens*, can synthesize diverse hopanoids (not including 2-methylhopanoids) when grown under strictly anaerobic conditions. Moreover, Rashby et al. (2007) report that copious quantities of 2-methylhopanoids are produced under anaerobic conditions mimicking those presumed to exist during Archaean time by *R. palustris*. As they state, "… because 2-MeBHPs may be produced by organisms that do not engage in oxygenic photosynthesis and because their biosynthesis does not require molecular oxygen, 2-methylhopanes cannot be used as *de facto* evidence for oxygenic photosynthesis" (p. 15102).

Side-chain degradation pattern. Brocks et al. (2003b, p. 4330) stated:

"The side-chain degradation pattern of the C30 to C36 2-methylhopane homologous series relative to the corresponding C30 to C36 3methylhopanes suggests that late Archaean cyanobacteria lived in an oxygenated micro-environment. The C30 to C36 2–methylhopane series has in all analyzed samples a characteristic even-over odd carbon number predominance. The elevated abundance of the C32-homologue relative to C31 and C33 indicates oxidative side-chain cleavage of a bacteriohopanetetrol to a C33

carboxylic acid and subsequent decarboxylation under non-reducing conditions."

The even-over-odd carbon number predominance of 2-methylhopanes relative to 3-methylhopanes can also be explained by anaerobic processes. So and Yung (1999) isolated an anaerobic sulphate-reducing bacterial strain from a petroleum-contaminated sediment that metabolizes straight-chain saturated alkanes with a similar two-carbon removal process. If grown on pure C_{even} alkanes, their total fatty acids were predominantly even in carbon number, while if grown on C_{odd} alkanes, their total fatty acids were predominantly odd in carbon number. Although this example is from the degradation of alkanes, similar chemistry ought to work for the degradation of isoprenoid side chains. While post-depositional degradation would affect side-chains of the 2- and 3-methylhopane fractions equally, it is clear that anaerobic organisms have a variety of biochemical pathways involving the addition of small carbon compounds that can produce such even/odd patterns, the complexity and mechanisms of which have yet to be resolved (see Berthe-Corti & Fetzner (2002) and Aeckersberg *et al.* (1998) and references therein).

Sterols. Sterols and their diagenetic derivatives, steranes, are potentially more relevant to the oxygen question. In yeast, the modern biosynthetic pathway to ergosterol (the simplest sterol required for strictly anaerobic growth (Andreasen & Stier 1953)) is a long chain of biochemical reactions that use the following three O_2-dependent enzymes: (*i*) squalene monooxygenase, (*ii*) lanosterol 14--methyl demethylase cytochrome P450, and (*iii*) sterol-4-methyl oxidase (Risley 2002). Figure 3 shows the first biosynthetic steps of this pathway, from the linear squalene molecule to lanosterol. Molecular oxygen is first used by enzyme (*i*) to form an epoxide bridge between the C2 and C3 carbons in the linear squalene molecule, which is then cyclized and converted to lanosterol as shown, leaving the oxygen as an hydroxyl on C3. To convert lanosterol to ergosterol, enzymes (*ii*) and (*iii*) use oxygen to remove three methyl groups, two on C4 and one on C14.

Because of the inertness of the C-H bond in hydrocarbons and the resonance energy of aromatic compounds, it is often assumed that oxygen must be involved in the activation, rearrangement, and metabolism of molecules like these. For demethylation, oxygen is used to introduce hydroxyl groups by replacing one of the carbon-bound hydrogens, after which other enzymes can proceed through a series of steps including dehydrogenation and eventual decarboxylation (see Darnet and Rahier (2003) for a recent overview of the oxidative mechanism for enzyme (*iii*)). It was long assumed that a direct hydrogen atom transport (HAT) reaction, where an activated radical removed a stably-bound H atom from a stable carbon subtrate leaving a reactive intermediate, was impossible under anaerobic conditions (see Suflita et al. (2004)for an excellent review). As noted below, this is no longer the case.

Goldfine (1965) recognized over 40 years ago that many anaerobic enzymes have been replaced by aerobic equivalents. Raymond and Blankenship (2004) discovered that, of the 473 reactions using O_2 as a substrate, there were more than 80, in at least 20 metabolic pathways, for which there was a direct anaerobic-to-aerobic substitution. Hence, the substitution of an O_2-dependent enzyme for an anaerobic one appears to be a common evolutionary occurrence. In at least one lipid pathway, that of heme/chlorophyll biosynthesis, 3 out of 17 steps were replaced on a 1-to-1 basis with oxygen-dependent enzymes; two of these are in the 9-step heme portion of the pathway. If 3 swaps happened in only 17 steps in the chlorophyll pathway, it is not unreasonable to suggest that 4 swaps might have happened in the >25 step sterol pathway.

Most enzymes involved in the sterol synthesis pathway are membrane-bound. Rather than being handed directly from one enzyme to another, substrates passively diffuse within this membrane layer between enzymes, which do not need to interact directly with each other. Hence, from an evolutionary perspective, it is easy to modify intermediate steps within a long biosynthetic pathway of this sort without "rebuilding" the entire chain from scratch. Squalene monooxygenase might have been a late addition to this pathway, and the cyclase enzyme could have evolved its specificity to 2,3-oxidosqualene sometime *after* the Great Oxygenation Event.

The question then focuses on whether or not there are plausible anaerobic substitutes for the steps catalyzed by the oxygen-dependent enzymes (*i*), (*ii*) and (*iii*) listed above, particularly the HAT reactions. The answer appears to be yes, and the relevant discoveries were triggered from

two surprising sources: economic geology and the discovery of anaerobic methane oxidation. Prior to 1990, it was thought that aerobic processes were the major cause of petroleum degradation in nature, yet it has since become clear from the study of deep oil reservoirs that extensive biological degradation occurred when sulphate-bearing (but anoxic) waters made contact with the hydrocarbons. A variety of sulphate-reducing bacteria were eventually implicated (Aeckersberg et al. 1991; Aitken et al. 2004; Rueter et al. 1994), and a series of previously unknown but powerful biochemical pathways were discovered (Boll et al. 2002; Widdel & Rabus 2001). Despite the stability and inertness of C-H and C-C bonds, these bacteria are able to perform all structural rearrangements needed to oxidize saturated and aromatic hydrocarbons to CO_2 anaerobically, including demethylation. Although, due to environmental and health concerns, the major biochemical work has been focused on the aromatic hydrocarbons like benzene, fully saturated hydrocarbons like alkanes and isoprenoids like pristane are also degraded and altered (Bonin et al. 2004; Suflita et al. 2004). A comparison of relevant bond energies for the first hydrogen atom extraction in the radical formation process RH R• + H• is illuminating; removal of an H atom from benzene requires ~113 kcal $mole^{-1}$, compared with only 105 and 101 from methane and ethane, respectively. The example of anaerobic methane oxidation, done by an archaeon working in concert with a sulphate-reducing bacterium (Orphan et al. 2001; Orphan et al. 2002), shows that these direct HAT reactions are indeed possible even without participation of electrons in adjacent orbitals, as may happen in aromatic compounds.

We will consider possible anaerobic precursors for the squalene monooxygenase (*i*) and demethylation (*ii, iii*) enzymatic steps separately next.

Squalene Monooxygenase. Brocks et al. (2003b) refer to the kinetic work of Jahnke & Klein (1983) on the squalene monooxygenase from the yeast *Saccharomyces cerevisiae* to argue that O_2 concentrations equivalent to 1% of the present atmospheric level would have been required for Archaean sterol synthesis; these levels would either demand the presence of photosynthetic oxygen or operate at the bottom of a melting, peroxide-rich ice sheet noted earlier.

However, the evidence permits alternative interpretations. First, the putative Archaean steranes do not have a hydroxyl group on the C3 carbon, which is the oxygen fingerprint of the squalene monooxygenase enzyme. Diagenesis would have removed the hydroxyl group had it originally been present, but without it there is no physical evidence that the squalene monooxygenase enzyme was ever involved in the formation of the particular sterol biomarkers in question. Although a clever organic geochemist might be able to infer indirectly whether or not a functional group was once present on the C3 carbon, to our knowledge no evidence of this sort has yet been reported. Using the Jahnke & Klein (1983) O_2 constraint for squalene monooxygenase as a paleoenvironmental indicator is an example of extrapolating the modern biosphere back nearly 3 billion years, and must be done with caution.

Nevertheless, it is worth considering the possibility that the Archaean steranes might have originally been sterols, and to determine if there are plausible anaerobic alternatives to get the hydroxyl on the C3 carbon. As noted by Fisher and Pearson (2007), it is not possible to hydroxylate the linear squalene molecule prior to cycilization and have the OH group wind up on the proper carbon, so an anaerobic hydroxylation step of this sort most likely came after the cyclization reaction. Conversion to the present epoxide-based sterol synthesis pathway would have happened *after* O_2 became abundant by an enzyme swap. This scenario is made more plausible by the fact that the oxidosqualene cyclase in eukaryotes evolved from a larger class of less substrate-specific bacterial squalene-hopene cyclase enzymes (Fischer & Pearson 2007; Pearson et al. 2003). Squalene cyclization reactions are highly exothermic, and the presence or absence of the oxygen in the epoxide moiety has little to do with the reaction kinetics (Fischer & Pearson 2007; Wendt et al. 1997). It also appears that the resulting stereochemical conformation of the product is controlled by how a methyl group on C_8 is positioned (Fischer & Pearson 2007), not by the configuration of the epoxide moiety as was once thought. These bacterial enzymes will even today cyclize either squalene or 2,3-oxidosqualene whereas the more specific eukaryotic version accepts only the 2,3-oxidosqualene.

For this scenario to work, there must have been an anaerobic hydroxylase capable of removing a C3 hydrogen from the sterene and

replacing it with hydroxyl. There are several possibilities for this. As noted above, this could be done by one of the direct sulphate-driven hydrogen atom extraction processes (RH R• + H•) (Bonin et al. 2004; Suflita et al. 2004). Extraction energies for this would be well below that required for benzene (~113 kcal $mole^{-1}$), a reaction that does proceed anaerobically. Alternatively, Kniemeyer and Heider (2001) purified and characterized a molybdenum/iron-sulphur /heme enzyme, ethylbenzene dehydrogenase, that catalyzed the anaerobic hydroxylation of the $-CH_2-$ of ethylbenzene. The reaction puts an oxygen atom from water onto the substrate as a hydroxyl while reducing an electron acceptor. Kniemeyer and Heider report similar activity for other related substrates, indicating that this is simply the first of a potentially large family dehydrogenase enzymes that can activate otherwise stable hydrocarbons without the use of O_2.

Demethylation steps (enzymatic reactions (ii) and (iii) above). To understand the mechanism for the oxidative removal of methyl groups, Darnet and Rahier (2003) developed a micelle-based system for characterizing the enzymological properties of the yeast sterol-C4-methyl-oxidase (enzyme *iii*), and using various mutants and information from the genome were able to confirm the reaction scheme. O_2, NADH, and cytochrome B_5 are used in the first step, converting the C4--methyl groups into the alcohol, $-CH_2OH$, which they were able to isolate from the micelles as a stable intermediary product. The same enzyme then catalyzes the removal of two more hydrogens to form the acetate, -COOH, presumably by the addition of the remaining oxygen not used in the first step. The subsequent removal of the carbon by the enzyme 4CD does not require oxygen, but it does involve the temporary conversion of the hydroxyl on C3 mentioned earlier into a ketone, making its presence essential for this particular O_2-dependent demethylation pathway. In yeast, this same enzyme removes both of the C4 methyl groups.

In contrast, Boll et al. (2002) review some completely anaerobic pathways for demethylation in sulphate and nitrate reducing bacteria. The best characterized of these is for the metabolism of toluene, which involves the initial addition of a fumarate co-substrate to the methyl group. A novel glycyl radical enzyme, (R)-benzylsuccinate synthase, removes a hydrogen from the methyl group and adds the fumarate to the methyl radical to yield benzylsuccinate. (This is the difficult activation step that avoids the use of molecular oxygen.) Following this, the benzylsuccinate is converted to benzoly-CoA and succinate via β-oxidation, and the succinate is recycled to fumarate via another enzyme, succinate dehydrogenase. As Boll et al. note, "*Thus, the overall pathway affords an oxygen-independent six electron oxidation of the methyl group of toluene to the carbonyl-CoA group of benzoyl-CoA*." These are representatives of a family of previously unknown enzymes that can activate of internal carbons by the addition of products that can be oxidized by conventional β-oxidation pathways, side-stepping the need for activation by molecular oxygen (Boll et al. 2002). They also observe further that variants of the fumarate pathway appear to be 'everywhere' in the anaerobic metabolism of hydrocarbons in a large variety of sulphate and nitrate reducing organisms. Hence, a similar mechanism tailored to the removal of the C4 (and C14) methyl groups of lanosterol is not unreasonable, and in addition would not need an adjacent hydroxyl group on C3 to proceed, which the oxidative pathway of sterol-C4-methyl-oxidase (enzyme (*iii*) above) apparently does require. As the complete genome sequences are known for several of these sulphate-reducing organisms as well as the yeast, *S. cervisiae*, it may be possible to recreate completely anaerobic sterol biosynthesis by re-introducing these fumarate pathways into yeast or other model eukaryotes.

An additional category of powerful anaerobic enzymes capable of doing structural rearrangements on hydrocarbon skeletons includes the radical-SAM reactions (Imlay 2006) (Supp. Fig. 1). This super-family of enzymes can produce S-adenosylmethionine (SAM) radicals energetic enough to catalyze the removal of a single hydrogen atom from a carbon backbone. In all of these enzymes studied to date, the reaction mechanism appears to proceed by causing an exposed iron in a [4Fe-4S] cluster to move from tetrahedral-like coordination to the octahedral geometry. In the process, the sulphur atom bound to the adenosylmethionine moiety is released in a highly activated form, and can be focused enzymatically to perform dehydrogenation, demethylation, or other structural rearrangements. Oxygen, however, poisons the Fe-S clusters by oxidizing the iron atoms in all of these enzymes.

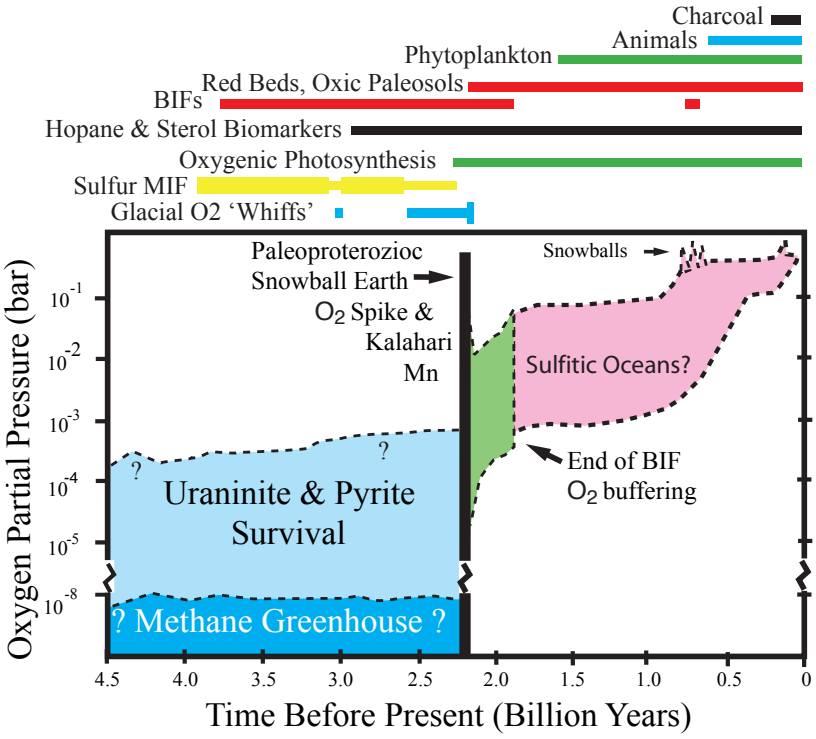

Fig. 4. History of Earth's oxygenation, showing important constraints described in the text. Adapted from Kirschvink & Weiss (Kirschvink & Weiss 2002). Question marks indicate uncer tainty in the relative levels of oxygen present.

In summary, although enzymes with the proper protein scaffolding have not yet been discovered, the biosphere has preserved the chemistry necessary to perform anaerobic sterol synthesis. The absence of the enzymes themselves is unsurprising given the rapidity with which the scaffolding portion of enzymes evolve compared to the functional groups and the selection pressure to swap entire enzymes for ones that are O_2 tolerant. Hence, we remain skeptical that the removal of these methyl groups can be used as a firm constraint on the existence of abundant oxygen supplies in the Archaean oceans.

5. WHEN DOES GEOLOGICAL EVIDENCE DEMAND THE PRESENCE OF OXYGENIC PHOTOSYNTHESIS?

The question of when oxygenic photosynthesis and environmental free oxygen first arose can be addressed from two directions. One could, on the grounds of uniformitarianism, assume that Earth system processes resembled modern processes as far back as the slightest bit of geological evidence suggests a kinship; one could assume that any oxidation or biochemical process that involves oxygen today has always involved oxygen, and any evidence of such a process is therefore evidence for oxygenic photosynthesis. This has been done many times (e.g., Rosing & Frei 2004), and a great deal of elegant intellectual effort has been spent trying to explain how oxidation of Earth's surface could be delayed many hundreds of million years after the advent of oxygenic photosynthesis (e.g., Canfield 2005; Catling & Claire 2005; Fennel et al. 2005).

Alternatively, following MacGregor (1927), one could take a skeptical approach and, working forward from Earth's origin, question the essence of oxygenic photosynthesis until the rock record permits no alternative explanation. As with all dichotomies, the most correct answer probably lies in between. The skeptical approach is a fruitful one for identifying the limits of current scientific understanding and generating new hypotheses to direct testing of those limits, but a strictly skeptical approach would render it nearly impossible to investigate many historically contingent events.

Our skeptical hypothesis to explain the Great Oxygenation Event (Figure 4) rejects, for the reasons discussed above, the uniformitarian interpretation of the biomarker record and accepts

the direct geological indicators of the redox transition (using the redox tower indicated in Figure 1). In the Archaean, the presence of detrital uraninite, detrital pyrite, and sulphur MIF argue for a dominantly anaerobic surficial environment, kept clement by a combination of greenhouse gases including H_2O, CO_2, CH_4, and perhaps SO_2. The redox levels present in the environment were governed by reducing gases like CH_4, sulphate produced by photo-oxidation (Farquhar et al. 2001) and microbial disproportionation (Philippot et al. 2007) of S^o, volcanogenic SO_2, and ferric iron produced by anaerobic iron-oxidizing photosynthetic bacteria (Kappler et al. 2005; Walker 1987; Widdel et al. 1993).

During glacial intervals, such as the Pongola at ~2.9 Ga and the Huronian at ~2.5-2.3 Ga, H_2O_2 would accumulate in ice and be released in the basal meltwaters. O_2 released by H_2O_2 disproportionation could then act as a local agent for oxic poisoning and drive the evolution of oxygen-mediating enzymes. Observed decreases in sulphur MIF associated with these glacial intervals may be products of oceanographic changes. Both enhanced physical mixing, associated with the larger pole-equator thermal gradient present in Icehouse conditions (Kopp et al. 2005), and the presence of more oxidizing electron acceptors, associated with the glacial peroxide source, would lead to greater mixing of oxidized and reduced sulphur reservoirs and thus diminish the MIF preserved in the sedimentary record.

Several redox sensitive trace elements have been used in attempt to constrain paleoredox conditions, notably U, Mo, and Re (e.g., Anbar et al. 2007; Rosing & Frei 2004). Uranium is soluble in its oxidized form and insoluble in its reduced form, so evidence for uranium mobility has been taken as evidence for oxidizing conditions. The redox potential of the U(VI)/U(VIII) couplet is, however, comparable to that of the ferrous/ferric couplet, and so does not provide much additional constraint on oxygen levels. Mo(IV) similarly forms insoluble sulphide minerals in its reduced form and is soluble in its oxidized Mo(VI) state. Due to the stability of MoS_2, increased Mo input through oxidative weathering provides a better constraint on redox conditions, but the redox potential of the MoS_2/ Mo(VI) couplet is still below that of other redox sensitive metals, such as Mn(II)/Mn(IV), below that of the P_{870} photosystem complex of purple bacteria, and well below that of H_2O/O_2 (Supplemental Table 1).

The oldest evidence for an environment containing massive amounts of free molecular oxygen of which we are aware is the ~ 2.22 Ga Kalahari Manganese Member of the Hotazel Formation, Transvaal Supergroup, South Africa (Cairncross et al. 1997). As indicated on Figure 1 and in Supplemental Table 1, nitrate and molecular O_2 are the only environmentally significant oxidants capable of converting soluble Mn^{2+} into insoluble Mn^{+4}, and nitrate itself requires oxygen to form in the modern oceans. Hence, the deposition of the BIF-hosted manganese in this unit is a firm oxygen constraint. The Hotazel Formation was also deposited in the aftermath of the Makganyene Snowball Earth Event (Kirschvink et al. 2000; Kopp et al. 2005), the only confirmed low-latitude glaciation in the Paleoproterozoic (Evans et al. 1997; Hilburn et al. 2005).

It is hard to imagine a more dramatic step in biochemical evolution than the final tinkering with the manganese-calcium cluster in the oxygen evolving complex of photosystem II that led to the splitting of water and the release of molecular O_2. Novel innovations that confer large selective advantages to a species lead to their rapid, exponential growth, and the creation and dominance of ecological niches. Such a profound evolutionary innovation ought to have left a clearly legible mark on the planet. Two dramatic events punctuate Earth History in the critical period around 2.3 Ga, when our skeptical interpretation suggests the first appearance of oxygenic photosynthesis: the Makganyene Snowball Earth and the deposition of the Kalahari Manganese Field. As we argued elsewhere (Kopp et al. 2005), the simplest explanation of these occurrences is that the sudden release of oxygen from the evolution and radiation of oxygenic phototrophs destroyed reduced greenhouse gases like methane and initiated Snowball conditions.

An additional implication of this scenario is that the formation of glacial peroxides is an essential step in the development of oxygen mediating enzymes, and ultimately oxygenic photosynthesis. Earth-like planets that orbit too close to their parent star for ice to form are therefore unlikely to evolve the aerobic metabolism essential for animal life.

6. CONCLUSIONS

As noted by Liang et al. (2006) production and accumulation of hydrogen peroxide on the surface of polar ice caps provides a straightforward mechanism for generating trace but substantial concentrations of H_2O_2 and free O_2 in the ocean below melting glaciers, away from the lethal influence of UV radiation. This environment is capable of driving the evolution of oxygen mediating enzymes, and paving the way for the evolution of the oxygen-evolving cluster of PS-II.

We find no chemical requirement for molecular oxygen in the biosynthesis of the lipid biomarkers of presumed Archaean age that cannot be met by known anaerobic biochemical mechanisms. As the anaerobic enzymes that perform analogous chemical steps to oxygen-requiring ones use redox-sensitive metal cofactors that are poisoned by oxygen (Imlay 2006), there is intense evolutionary pressure to swap them out for those that are not oxygen sensitive. As 3 such substitutions in 17 enzymatic steps are documented in the biosynthesis of chlorophyll, we argue that 4 substitutions in a >25 step sterol synthesis pathway are not unreasonable.

The evolution of the oxygen-releasing complex of PS-II as measured by environmental redox indicators is correlated with the Makganyene Snowball Earth and the deposition of the Kalahari Manganese Field. We suggest that the destruction of reduced greenhouse gases like methane(Pavlov et al. 2000) did not take 400 million years, but was rapid enough to trigger the Makganyene Snowball (e.g., Kopp et al. 2005).

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QUESTIONS AND ANSWERS FROM THE DISCUSSION

1. Martin

I have also been following some of these microbiology papers showing the utility of microbiological biochemistry to generate exactly these marker molecules (hopanes) under strictly anaerobic conditions in laboratory experiments. This provides clear-cut evidence that they can make the molecules in question without the presence of oxygen, calling generally into question the value of these markers as positive evidence for either the presence of oxygen or, and this is my main point, of eukaryotes.

Kirschvink. We agree.

Martin. Another comment is that the 'tree of life' derived from ribosomal RNA is *extremely* contentious whereas we have direct evidence for the history of life in the palaeontological record and the geochemical record. What is the motto of the Royal Society?

Ellis. Nullius in verba - Nothing in words.

Martin. Which translates to: Just don't take my word for it.

Kirschvink. Like MacGregor, we agree entirely with this comment.

2. Simon Styring

Purple bacteria are purple and cyanobacteria are green because of the difference in the pigments, not in the manganese cluster or the catalytic sites. The purple pigments do not work at very high redox potentials so they cannot oxidize water. The green pigments, chlorophylls, work at very high redox potentials and P680 is the driver for the Mn cluster. There was no Mn cluster before there was a driver. The highly oxidizing chlorophylls must have been around first. There must have been something in the food chain available for the organism that purple bacteria could not oxidize. One option would be that it could be MnII itself.

Kirschvink. Mn^{+2} would certainly be abundant in the Archaean oceans, as it is emitted from hydrothermal vent systems at a 1:5 molar ratio with Fe^{+2}. We will deal with this in a future manuscript.

3. Roger Summons

One of the earliest persons you cited was Guy Ourisson who was one of the pioneers of the study of hopanoids in rocks and organisms. As far as I know he did not work on Precambrian organic matter but he wrote a paper about it. His point was that there are no biomarkers that are specific to Proterozoic rock because once organisms invent a useful piece of biochemistry they don't forget about it. So far as he knew there were no biochemical pathways that had gone extinct to the point that you could use them as specific markers for Proterozoic or older rocks. Your use of the chlorophyll analogy just proves the point about sterols, because we know that there are some anoxygenic steps in that pathway because they are extant now. As far as we know there are no analogous anoxygenic steps in the sterol biosynthetic pathway, so Occam's razor and parsimony really force us to believe that there never was an anaerobic pathway to sterols.

Kirschvink. Well, we disagree slightly. What Ourisson actually meant was that reaction *mechanisms* are unlikely to go extinct. Most enzymes combine a reaction center (where the interesting chemistry happens) with a protein scaffolding that holds the reactant molecules in the proper position so that the reaction can go properly. That scaffolding evolves rapidly, which is why we can isolate families of enzymes that do similar chemistry but on slightly different substrates. We are arguing here that the biosphere has not forgotten these Archaean mechanisms – they still exist in the anaerobic world – but that some specific enzymes that are particularly sensitive to O_2 poisoning have been updated with enzyme systems that are not so sensitive, or actually use O_2 as a substrate. We mention this in the new summary for section 5.

4. Oliver Morton

You showed a chart showing the average temperature throughout the Archaean. What do you think was driving the temperature down to the point where you got these glaciations?

Kirschvink. This is a backwards question – with the Sun at 30% of the present luminosity, we need to understand what is keeping the planet WARM, not cold!

Table S1: Midpoint potentials of relevant redox couplets at pH 7

Redox couplet	**E (mV)**
CO_2 / CH_4 (a,b)	-230
SO_4^{2-} / HS^- (c)	-217
$Fe(OH)_{3\ (ferrihydrite)}$ / Fe^{2+} (c)	-5
$UO_2(CO_3)_2^{2-}$ / $UO_{2\ (uraninite)}$ + CO_2 (a,d)	-18
NO_3^- / NH_4^+ (c)	+366
MoO_4^{2-} / $MoS_{2\ (molybdenite)}$ (e)	+411
P_{870}/P_{870}^+ (purple bacteria)	~+450
$MnO_{2\ (pyrolusite)}$ / Mn^{2+} (c)	+490
NO_3^- / N_2 (c)	+717
O_2 / H_2O	+815
P_{680}/P_{680}^+ (Photosystem II)	~+1100

a. Calculated for pCO_2 = 100 mbar. b. Calculated for pCH_4 = 1 mbar. c. Calculated with all aqueous reactants at 1 mM. d. Calculated with dissolved U species at 20 nM. e. Calculated with dissolved Mo species at 10 nM. (All reactants not specifically noted are at standard state. Thermodynamic constants from Drever (1997), Langmuir (1978), Hepler (1969), Arnórsson (1985) and Bard et al. (1985). Photosystem potentials are from White (2000).)

Arnórsson, S. & Ívarsson, G. 1985 Molybdenum in Icelandic geothermal waters. *Contributions to Mineralogy and Petrology* 90, 179-189.

Bard, A. J., Parson, R. & Jordan, J. 1985 *Standard Potentials in Aqueous Solution*. Monographs in Electroanalytical Chemistry and Electrochemistry. New York: CRC Press.

Drever, J. I. 1997 *The Geochemistry of Natural Waters: Surface and Groundwater Environments*. Englewood Cliffs, NJ: Prentice Hall.

Hepler, L. G. 1969 Thermodynamic properties of ruthenate and molybdate ions. *Canadian Journal of Chemistry* 47, 3469-3470.

Langmuir, D. 1978 Uranium Solution-Mineral Equilibria at Low-Temperatures with Applications to Sedimentary Ore-Deposits. *Geochimica Et Cosmochimica Acta* 42, 547-569.

White, D. 2000 *The Physiology and Biochemistry of Prokaryotes*. New York: Oxford University Press.

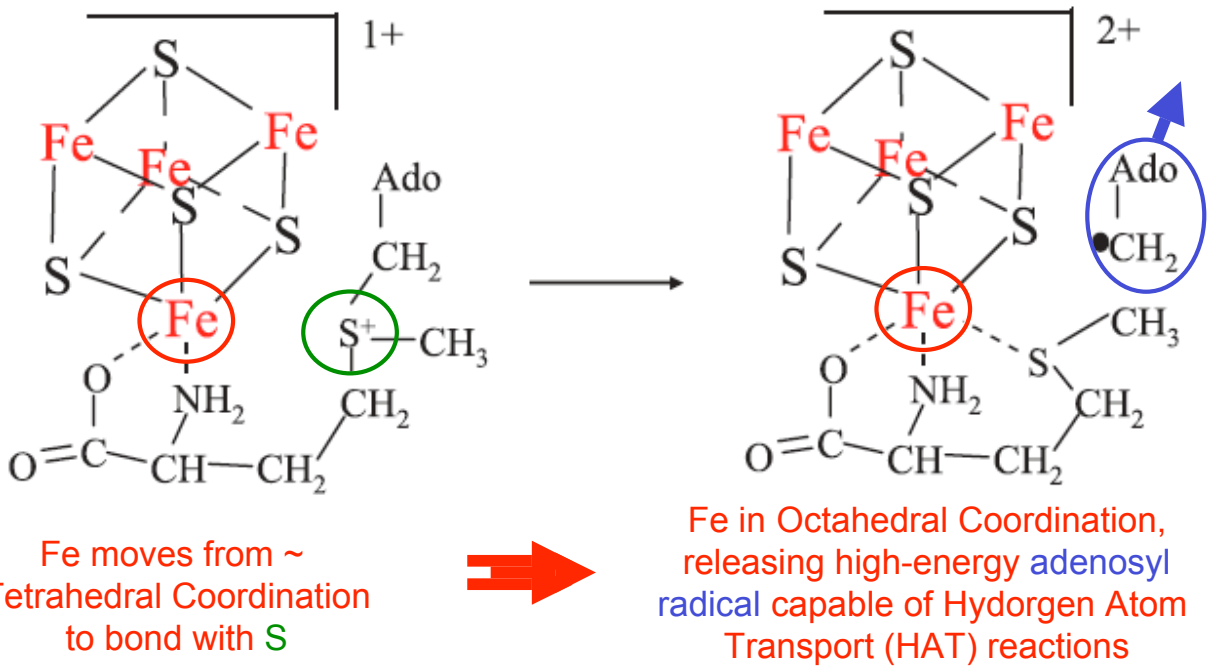

Figure S1. Reaction scheme leading to the production of high-energy S-adenosylmethionine radicals (Radical SAM reaction), adapted from Imlay (2006) and Chen et al., (2003). The high-energy radical is formed when the 4Fe-4S cluster changes configuration so that one of the Fe atoms in ~tetrahedral coordination (circled in red) moves to form a bond with an adjacent sulfur atom (green circle), merging towards octahedral coordination. The result is the controlled release of a high-energy adenosyl radical (blue circle) capable of performing direct hydrogen atom transfer (HAT) reactions, as described in the text.

Chen, D. W., Walsby, C., Hoffman, B. M. & Frey, P. A. 2003 Coordination and mechanism of reversible cleavage of S-adenosylmethionine by the [4Fe-4S] center in lysine 2,3-aminomutase. *Journal of the American Chemical Society* 125, 11788-11789.

Imlay, J. A. 2006 Iron-sulphur clusters and the problem with oxygen. *Molecular Microbiology* 59, 1073-1082.